METHOD FOR RECOMBINANT ADENO-ASSOCIATED VIRUS-DIRECTED GENE THERAPY

Background of the Invention

Adeno-associated virus (AAV) is a replication-deficient parvovirus, the genome of which is about 4.6 kb in length, including 145 nucleotide inverted terminal repeats (ITRs). The single-stranded DNA genome of AAV contains genes responsible for replication (rep) and formation of virions (cap).

When this nonpathogenic human virus infects a human cell, the viral genome integrates into chromosome 19 resulting in latent infection of the cell. Production of infectious virus and replication of the virus does not occur unless the cell is coinfected with a lytic helper virus such as adenovirus or herpesvirus. Upon infection with a helper virus, the AAV provirus is rescued and amplified, and both AAV and helper virus are produced.

AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells. Various groups have studied the potential use of AAV in the treatment of disease states.

Studies of recombinant AAV (rAAV) *in vitro* have been disappointing because of low frequencies of transduction; incubation of cells with rAAV in the absence of contaminating wild-type AAV or helper adenovirus is associated with little recombinant gene expression [D. Russell et al, Proc. Natl. Acad. Sci. USA, 91:8915-8919 (1994); I. Alexander et al, J. Virol., 68:8282-8287 (1994); D. Russell et al, Proc. Natl. Acad. Sci. USA, 92:5719-5723 (1995); K. Fisher et al, J. Virol., 70:520-532 (1996); and F. Ferrari et al, J. Virol., 70:3227-3234 (1996)]. Furthermore, integration is inefficient and not

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directed to chromosome 19 when *rep* is absent [S. Kumar et al, J. Mol. Biol., 222:45-57 (1991)]. AAV transduction is substantially enhanced in the presence of adenovirus because the single-stranded rAAV genome is converted to a non-integrated, double-stranded intermediate which is transcriptionally active [K. Fisher et al, J. Virol., 70:520-532 (1996); and F. Ferrari et al, J. Virol., 70:3227-3234 (1996)]. Adenovirus augments rAAV transduction through the expression of the early gene product E4 ORF6 [K. Fisher et al, J. Virol., 70:520-532 (1996); and F. Ferrari et al, J. Virol., 70:3227-3234 (1996)].

The performance of rAAV as a vector for *in vivo* models of gene therapy has been mixed. The most promising results have been in the central nervous system, where stable transduction has been achieved in postmitotic cells [M. Kaplitt et al, Nat. Genet., 8:148-154 (1994)]. Incubation of bone marrow cells *ex vivo* with rAAV results in some transduction, although stable and efficient hematopoietic engraftment has not been demonstrated in transplant models [J. Miller et al, Proc. Natl. Acad. Sci. USA, 91:10183-10187 (1994); G. Podsakoff et al, J. Virol., 68:5656-5666 (1994); and C. Walsh et al, J. Clin. Invest., 94:1440-1448 (1994)]. Administration of rAAV into the airway or the blood leads to gene transfer into lung epithelial cells [K. Fisher et al, J. Virol., 70:520-532 (1996); and T. Flotte et al, Proc. Natl. Acad. Sci. USA, 90:10613-10617 (1993)] and hepatocytes [K. Fisher et al, J. Virol., 70:520-532 (1996)] respectively; however, transgene expression has been found to be low unless adenovirus is present [K. Fisher et al, J. Virol., 70:520-532 (1996)].

What is needed is a method of improving rAAV-mediated gene transfer.

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Summary of the Invention

The present invention provides a method of improving expression of a selected gene delivered to an animal via recombinant AAV. The method involves introducing a recombinant AAV vector comprising a desired transgene into a muscle cell in the absence of a helper virus. The vector may be administered into cardiac, smooth, or, preferably, skeletal muscle.

In one preferred embodiment, the rAAV-delivered transgene encodes a secretable and/or diffusable product which is therapeutically useful. In this embodiment, the transgene product may have therapeutic effect on sites at a distance from the delivery site. In another embodiment, the transgene encodes a non-secretable product (e.g., a dystrophin polypeptide) for which delivery to the muscle is desired (e.g., for treatment of muscular dystrophy).

In another aspect, the present invention provides a method of treating an animal with hemophilia. The method involves administering into the muscle of the animal a recombinant adeno-associated virus comprising the gene encoding factor IX and sequences which regulate expression of the gene.

In yet another aspect, the invention provides a method of treating an animal with atherosclerosis. The method involves administering into the muscle of the animal a recombinant adeno-associated virus comprising the gene encoding ApoE and regulatory sequences capable of expressing said gene.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

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<u>Brief Description of the Drawings</u>

Fig. 1 is a schematic illustration showing the
linear arrangement of AAV.CMVLacZ (4883 bp). The
relevant elements include AAV ITRs (solid black boxes),
5   CMV promoter (hatched arrow), SV40 intron and
polyadenylation signal (open boxes), and lacZ DNA (shaded
box). The location of a cDNA probe that can be used to
detect the internal BamHI fragment, as well as full-
length vector, is also shown.

10   Fig. 2A is a schematic illustration of the linear
arrangement of AAV.CMVLacZ concatomer. The relevant
landmarks include AAV ITRs (hatched boxes), CMV
enhancer/promoter (solid black arrow), SV40 intron and
polyadenylation signal (open boxes), and lacZ cDNA
15   (shaded box). AAV.CMVLacZ monomer is shown joined
according to a direct end-to-end ligation mechanism
(labeled j) at the ITRs. Therefore, in the cartoon two
copies of the AAV ITR are present at the junction.

Fig. 2B is a schematic illustration showing an
20   amplified view of the junction domain. Relevant
landmarks are as indicated in Fig. 3A above. Horizontal
arrows indicate the location and direction of PCR primers
used to amplify across the provirus junction. Primer 005
is a sense-strand primer. Primers 013 and 017 are
25   antisense-strand primers.

Fig. 3 is a schematic illustration showing the
predicted PCR product assuming a direct end-to-end tandem
ligation of monomer AAV.CMVLacZ genomes. Two complete
ITRs (shaded box) with their respective "FLOP" and "FLIP"
30   orientation are shown at the junction (labeled j). The
CMV promoter (solid black box) and polyadenylation signal
(open box) are also indicated. The PCR cloning site in
pCRII is flanked by EcoRI sites as shown. The location
of three SnaBI sites positioned within the PCR product
35   are also shown. Primers 005 and 013 also indicated.

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Fig. 4A, in conjunction with Figs. 4B-4G, illustrates the structure of PCR products that map to the head-to-tail junction of AAV.CMVLacZ concatomers of Fig. 4A. Fig. 4A shows the predicted PCR product assuming a

5   direct end-to-end tandem ligation of monomer AAV.CMVLacZgenomes. Two complete ITRs (shaded box) with their respective "FLOP" and "FLIP" orientation are shown at the junction (labeled j). The CMV promoter (solid black box) and polyadenylation signal (open box) are also

10   indicated.

Fig. 4B illustrates the structure of the PCR product from Clone 3.

Fig. 4C illustrates the structure of the PCR product from Clone 8. Clone 8 is nearly identical in size to

15   clone 3, but contains a different rearrangement of the ITR junction.

Fig. 4D illustrates the structure of the PCR product from Clone 5.

Fig. 4E illustrates the structure of the PCR product

20   from Clone 2.

Fig. 4F illustrates the structure of the PCR product from Clone 6.

Fig. 4G illustrates the structure of the PCR product from Clone 7.

25   Fig. 5A characterizes the activation of cytotoxic T lymphocytes directed against adenoviral antigen as well as *lacZ*. This is an analysis of lymphocytes harvested from Group 1 of Example 5.

Fig. 5B characterizes the activation of cytotoxic T

30   lymphocytes directed against adenoviral antigen as well as *lacZ*. This is an analysis of lymphocytes harvested from Group 2 of Example 5.

Fig. 5C characterizes the activation of cytotoxic T lymphocytes directed against adenoviral antigen as well

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as *lacZ*. This is an analysis of lymphocytes harvested from Group 3 of Example 5.

Fig. 6A shows the activation of T lymphocytes in response to different antigens including β-galactosidase, purified AAV, or adenovirus, for each of Groups 1-4 of Example 5. Activation is demonstrated by the secretion of IFN-γ representing the TH1 subset of T cells.

Fig. 6B shows the activation of T lymphocytes in response to different antigens including β-galactosidase, purified AAV, or adenovirus, for each of Groups 1-4 of Example 5. Activation is demonstrated by the secretion of IL-10 representing the TH2 subset of T cells.

Fig. 7A illustrates results from an enzyme linked immunosorbent assay (ELISA), showing the development of antibodies directed against β-galactosidase in the various groups of example 5.

Fig. 7B illustrates results from an enzyme linked immunosorbent assay (ELISA), showing the development of antibodies directed against adenovirus type 5 in the various groups of Example 5.

Fig. 8 is a graph of plasma concentration of hF.IX in C57BL/6 mice as a function of time following IM injection of 2×10^{11} rAAV-hF.IX vector genomes/animal (n=4).

Fig. 9 is a graph showing circulating antibody against human F.IX as a result of im injection of rAAV-hF.IX in C57BL/6 mice. The time course of anti-hF.IX antibody concentration in plasma after injection with 2×10^{11} vector genomes/animal (n=3) was determined by ELISA using mouse MAb anti-hF.IX [Boehringer Mannheim] as a standard. Each line represents an individual animal.

Fig. 10 illustrates plasma concentration of hF.IX in three mice as a function of time post-injection. Each symbol represents a different animal. The fourth animal

in this experiment died 5 weeks post-injection following
traumatic phlebotomy.

    Fig. 11 is a graph illustrating plasma concentration
of hF.IX in four Rag-1 mice as a function of time post-
injection with rAAV-hF.IX. Each symbol represents a
different animal.

    Fig. 12 is a schematic diagram of a head-to-tail
concatamer of the rAAV present in transduced cells.

    Fig. 13 is a schematic diagram illustrating the
construction of AV.CMVApoE.

Detailed Description of the Invention

    The present invention provides a method for adeno-
associated virus (AAV) mediated muscle-directed gene
transfer which provides high level and stable transgene
expression in the absence of helper virus or exogenous
helper molecules. Particularly, this method involves
introducing a recombinant AAV carrying a desired
transgene into a muscle cell. Desirably, the rAAV vector
is injected directly into cardiac, skeletal, or smooth
muscle and the transgene encodes a secreted and/or
diffusable therapeutic product, such as a polypeptide or
an RNA molecule. However, the method of the invention is
similarly useful for administration of nucleic acids
encoding non-secreted, therapeutically useful products.

    Particularly, the inventors have discovered that
intramuscular injection of helper-free purified rAAV
(i.e., rAAV which is substantially free of contamination
with adenovirus or wild-type AAV) leads to highly
efficient transduction of muscle fibers leading to stable
and prolonged transgene expression. By helper-free is
further meant that the AAV is in the substantial absence
of helper virus or other exogenous helper molecules
(i.e., helper molecules not native to or normally present
in muscle cells). This is accomplished without

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significant inflammation or activation of immune
responses to the transgene product, despite the fact that
the product may be a neoantigen.

    The stability of transgene expression produced
5   according to the methods of this invention is
particularly impressive. Without wishing to be bound by
theory, this stability is believed to be due to the
highly inefficient chromosomal integration of AAV
proviral DNA in muscle cells in the absence of helper
10 virus or other exogenous helper molecules. Several
observations support this hypothesis. As described in
the examples below, analysis of Hirt extracts failed to
detect a double-stranded episomal form of the viral
genome. Southern analysis of total cellular DNA revealed
15 a discrete band when digested with an enzyme that has two
cleavage sites within the AAV vector, whereas no discrete
band was observed when the same DNA was digested with an
enzyme that does not have sites within the viral genome.

    Further DNA analysis focused on the formation of
20 concatamers and their structure. Previous studies of
lytic AAV infections have shown that the episomal
replication of rAAV proceeds through head-to-head or
tail-to-tail concatamers, while latent infections that
result in proviral integration are established as head-
25 to-tail tandem arrays [K.I. Berns, <u>Microbiol. Rev.</u>,
<u>54</u>:316-329 (1990); J.-D. Tratschin et al, <u>Mol. Cell.</u>
<u>Biol.</u>, <u>5</u>:3251-3260 (1985); N. Muzcyzska, <u>Current Topics</u>
<u>in Microbiology and Immunology</u>, <u>158</u>:97-129 (1993); S.K.
McLauglin et la, <u>J. Virol.</u>, <u>62</u>:1963-1973 (1988)]. The
30 data set forth herein demonstrate that muscle cells
transduced with rAAV vectors according to this invention
contain concatamers consisting of head-to-tail tandem
arrays with variable deletions of both ITRs, consistent
with a transduction mechanism involving integration of
35 rAAV provirus.

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Sequence analysis of the junctions created by rAAV
concatamers indicated consistent but variable deletions
of both ITRs. Fluorescent in situ hybridization (FISH)
analysis was consistent with single integration sites in
5   approximately 1 in 20 nuclei, while Southern analysis
indicated an average of 1 proviral genome per diploid
genome of the muscle fiber. Together these findings
indicate that the average concatamers minimally comprises
ten proviral genomes.

10   Another advantage of the method of the invention is
the surprising absence of inflammation upon
administration of therapeutic doses of vector. For
example, C57BL/6 mice injected with a lacZ AAV vector
failed to mount a humoral immune response to *E.coli* ß-

15   galactosidase despite the fact that vibrant anti-ß-
galactosidase antibodies were elicited in these animals
when a lacZ adenoviral vector was injected into skeletal
muscle. Thus, when the helper-free AAV vector was used
according to the method of this invention, immune

20   responses to the transgene were modulated. This
contrasts sharply with prior art methods of gene
transfer, such as those which employ naked plasmid DNA
[J.A. Wolff et al, Science, 247:1465-1468 (1990)] or
adenovirus-mediated gene therapy [S.K. Tripathy et al,

25   Nat. Med., 2:545-550 (1996)], which typically elicit
strong immune responses to the transgene. Thus, the
method of the invention provides a significant advantage
over other gene delivery systems, particularly with
respect to the treatment of chronic disorders that may

30   require repeated administrations.

I.   The Recombinant AAV

A recombinant AAV vector carrying a selected
transgene is used in the methods of the invention. In
addition to the transgene, the vector further contains

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regulatory sequences which control expression of the
transgene in a host cell, e.g., a muscle cell.

    Many rAAV vectors are known to those of skill in the
art and the invention is not limited to any particular

5   rAAV vector. For example, suitable AAV vectors and
methods of producing same are described in   U. S.
Patent No. 5,252,479; U. S. Patent No. 5,139,941;
International Patent Application No. WO94/13788; and
International Patent Application No. WO93/24641. One

10 particularly desired vector is described below.

      A.   <u>The AAV Sequences</u>

    Currently, a preferred rAAV is deleted of·all
viral open reading frames (ORFs) and retains only the
cis-acting 5' and 3' inverted terminal repeat (ITR)

15 sequences [See, e.g., B. J. Carter, in "Handbook of
Parvoviruses", ed., P. Tijsser, CRC Press, pp.155-168
(1990)]. Thus, the *rep* and *cap* polypeptide encoding
sequences are deleted. The AAV ITR sequences are about
143 bp in length. While it is preferred that

20 substantially the entire 5' and 3' sequences which
comprise the ITRs are used in the vectors, the skilled
artisan will understand that some degree of minor
modification of these sequences is permissible. The
ability to modify these ITR sequences while retaining

25 their biological functions is within the skill of the
art. See, e.g., texts such as Sambrook et al, "Molecular
Cloning. A Laboratory Manual.", 2d edit., Cold Spring
Harbor Laboratory, New York (1989).

    The AAV ITR sequences may be obtained from any

30 known AAV, including presently identified human AAV
types. The selection of the AAV type is not anticipated
to limit the invention. A variety of AAV types,
including types 1-4, are available from the American Type
Culture Collection or available by request from a variety

35 of commercial and institutional sources. Similarly, AAVs

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known to infect other animals may also be employed in the
vector used in the methods of this invention. In the
examples set forth herein, an AAV-2 is used for
convenience. Specifically, the 5' and 3' AAV ITR
5   sequences from AAV-2 flank a selected transgene sequence
and associated regulatory elements, as described below.

B.   The Transgene

The transgene sequence contained within the
rAAV vector is a nucleic acid sequence, heterologous to
10   the AAV sequence, which encodes an RNA or polypeptide of
interest. The transgene is operatively linked to
regulatory components in a manner which permits transgene
expression in muscle cells.

The composition of the transgene sequence will
15   depend upon the use to which the resulting vector will be
put. For example, one type of transgene sequence
includes a reporter sequence, which upon expression
produces a detectable signal. Such reporter sequences
include without limitation an *E. coli* beta-galactosidase
20   (*LacZ*) cDNA, an alkaline phosphatase gene and a green
fluorescent protein gene. These sequences, when
associated with regulatory elements which drive their
expression, provide signals detectable by conventional
means, e.g., ultraviolet wavelength absorbance, visible
25   color change, etc. Expression of such transgenes may be
used in methods for cell quantitation, cell
identification and the like.

A more preferred transgene sequence includes a
therapeutic gene which encodes a desired gene product.
30   These therapeutic nucleic acid sequences typically encode
products which, when administered to a patient *in vivo* or
ex vivo, are able to replace or correct an inherited or
non-inherited genetic defect or treat an epigenetic
disorder or disease.

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The method of the invention, which delivers the transgene to the muscle cells, is particularly well suited for use in connection with secreted therapeutic proteins, such as factor IX, useful in treatment of
5   hemophilia, or apolipoprotein (Apo) E, useful in treatment of atherosclerosis. However, other therapeutic gene products, particularly those which are secreted, may be readily selected by the skilled artisan. Examples of genes encoding secreted and/or diffusable products,
10 include, without limitation, cytokines, growth factors, hormones, differentiation factors, and the like, e.g., β-interferon (β-IFN), erythropoietin (epo), insulin, growth hormone (GH), and parathyroid hormone (PTH). These genes are useful for treatment of a variety of conditions,
15 including multiple sclerosis and cancer (β-IFN), anemia (epo), diabetes (insulin), small stature (GH), and osteoporosis (PTH). The method of the invention is also useful for delivery of genes encoding non-secreted products to the muscle. For example, the method of the
20 invention is anticipated to be useful in treatment of muscular dystrophies, by enabling delivery of a dystrophin gene [see, e.g., C. C. Lee et al, Nature, 349:334-336 (1991)] via a rAAV according to the method of the invention. The selection of the transgene is not
25 considered to be a limitation of this invention, as such selection is within the knowledge of the skilled artisan.

        C.   Regulatory Elements of the Vector

        In addition to the AAV ITR sequences and the transgene, the vector also includes regulatory elements
30 necessary to drive expression of the transgene in transduced muscle cells. Thus the vector desirably contains a selected promoter and enhancer (if desired) which is operatively linked to the transgene and located, with the transgene, between the AAV ITR sequences of the
35 vector.

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        Selection of the promoter and, if desired, the
enhancer, is a routine matter and is not a limitation of
the vector itself. Useful promoters may be constitutive
promoters or regulated (inducible) promoters, which will
5   enable controlled expression of the transgene. For
example, a desirable promoter is that of the
cytomegalovirus immediate early promoter/enhancer [see,
e.g., Boshart et al, Cell, 41:521-530 (1985)]. Other
desirable promoters include, without limitation, the Rous
10   sarcoma virus LTR promoter/enhancer and the inducible
mouse metallothienien promoter. Still other
promoter/enhancer sequences may be selected by one of
skill in the art.

        The vectors will also desirably contain nucleic
15   acid sequences which affect transcription or translation
of the transgene including sequences providing signals
required for efficient polyadenylation of the transcript
and introns with functional splice donor and acceptor
sites. A common poly-A sequence which is employed in the
20   exemplary vectors of this invention is that derived from
the papovavirus SV-40. The poly-A sequence generally is
inserted into the vector following the transgene
sequences and before the 3' AAV ITR sequence. A common
intron sequence is also derived from SV-40, and is
25   referred to as the SV-40 T intron sequence. Selection of
these and other elements desirable to control or enhance
gene expression are conventional and many such sequences
are known to those of skill in the art [see, e.g.,
Sambrook et al, and references cited therein].

30   The combination of the transgene,
promoter/enhancer and the other regulatory elements are
referred to as a "minigene" for ease of reference herein.
As above stated, the minigene is flanked by the 5' and 3'
AAV ITR sequences. Provided with the teachings of this

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invention, the design of such a minigene can be readily
accomplished by the skilled artisan.

An example of a rAAV, i.e., AAV.CMVLacZ, and
its use in the method of the invention is provided in the
5   examples below. As illustrated in Fig. 1, this exemplary
rAAV contains a 5'AAV ITR, a CMV promoter, an SV-40
intron, a LacZ transgene, an SV-40 poly-A sequence and a
3'AAV ITR. However, as stated above, the method of this
invention is not limited to use of any particular rAAV.

10   D.   Production of rAAV

The sequences employed in the construction of
the rAAV used with the method of this invention may be
obtained from commercial or academic sources based on
previously published and described materials. These
15   materials may also be obtained from an individual patient
or generated and selected using standard recombinant
molecular cloning techniques known and practiced by those
skilled in the art. Any modification of existing nucleic
acid sequences used in the production of the rAAV
20   vectors, including sequence deletions, insertions, and
other mutations may also be generated using standard
techniques.

Assembly of the rAAV, including the sequences
of AAV, the transgene and other vector elements, may be
25   accomplished using conventional techniques. One
particularly desirable technique is described in K. J.
Fisher et al, J. Virol., 70(1):520-532 (January, 1996),
which is incorporated by reference herein. However,
other suitable techniques include cDNA cloning such as
30   those described in texts [Sambrook et al, cited above],
use of overlapping oligonucleotide sequences of the AAV
genome, polymerase chain reaction, and any suitable
method which provides the desired nucleotide sequence.
Where appropriate, standard transfection and co-
35   transfection techniques are employed to propagate the

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rAAV viruses in the presence of helper viruses, e.g.,
adenoviruses deleted for E1 using techniques such as
$CaPO_4$ transfection techniques, and may be readily
selected by the skilled artisan. Other conventional
5   methods which may be employed in this invention include
homologous recombination of AAV viral genomes, plaquing
of viruses in agar overlay, methods of measuring signal
generation, and the like.

Desirably, the rAAV produced are purified to
10   remove any contaminating adenovirus or wild-type AAV. A
particularly desirable purification scheme is described
in K. J. Fisher et al, _J. Virol._, _70_(1):520-532 (January,
1996), which is incorporated by reference. However, one
of skill in the art can readily select other appropriate
15   purification means.

II.   Therapeutic Applications

Once a rAAV containing a desired transgene is
obtained, the vector is administered directly into an
animal's muscle. One advantage of the method of the
20   invention is that muscle is particularly well suited as a
site for production of secreted therapeutic products,
such as factor IX or apolipoprotein (Apo) E, among
others. Alternatively, the method of·the invention is
used to deliver a non-secreted gene product to the muscle
25   cells.

The rAAV vectors of the present invention may be
administered to a patient, preferably suspended in a
biologically compatible solution or pharmaceutically
acceptable carrier or delivery vehicle. A suitable
30   vehicle includes sterile saline. Other aqueous and non-
aqueous isotonic sterile injection solutions and aqueous
and non-aqueous sterile suspensions known to be
pharmaceutically acceptable carriers and well known to

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those of skill in the art may be employed for this
purpose.

The rAAV vectors of this invention are administered
in sufficient amounts to provide for integration and
5   expression of the selected transgene such that a
therapeutic benefit may be obtained without undue adverse
effects and with medically acceptable physiological
effects which can be determined by those skilled in the
medical arts. In a preferred embodiment, the rAAV are
10   injected directly into cardiac, skeletal, or smooth
muscle. One of skill in the art will also appreciate
that other methods of administration, e.g., intravenous
or intraarterial injection may also be utilized in the
method of the invention so long as the rAAV is targeted
15   to the muscle cells.

Dosages of the rAAV vector will depend primarily on
factors such as the condition being treated, the selected
transgene, the age, weight and health of the patient, and
may thus vary among patients. A therapeutically
20   effective dose of the rAAV of the present invention is
believed to be in the range of from about 1 to about 50
ml of saline solution containing concentrations of from
about 1×10^8 to 1×10^{11} particles/ml rAAV vector of the
present invention. Desirably, each dose contains at
25   least 10^9 particles rAAV. A more preferred human dosage
is about 1-20 ml saline solution at the above
concentrations. The levels of expression of the selected
transgene can be monitored by bioassay to determine the
route, dose or frequency of administration.
30   Administration of the rAAV may be repeated as needed.

The examples set forth below illustrate the
preferred methods for preparing the vectors and
performing the methods of the invention. These examples
are illustrative only and do not limit the scope of the
35   invention.

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Example 1 - Production of AAV.CMV*LacZ*

A recombinant AAV (rAAV) was generated in which the
rep and *cap* genes were replaced with a minigene
expressing *E. coli* β-galactosidase under the control of a
CMV promoter (AAV.CMVlacZ). AAV.CMVlacZ was produced by
using the cis-acting plasmid pAAV.CMVlacZ, which was
derived from psub201 [R. Samulski et al, J. Virol.,
61(10):3096-3101 (1987)]. Briefly, the plasmid was
transfected into 293 cells infected with E1-deleted
adenovirus [K.J. Fisher et al, J. Virol., 70:520-532
(1996)] and AAV *rep* and *cap* functions were provided by a
transacting plasmid pAAV/Ad [R. Samulski et al, J.
Virol., 63:3822-3826 (1989)]. Production lots of
AAV.CMVLacZ vector were titered according to genome
copies/ml as described [Fisher et al, J. Virol., 70:520-
532 (1996)].

The 5'-to-3' organization of the AAV.CMV*LacZ* genome
(4883 bp), includes:

the 5' AAV ITR (bp 1-173) obtained by PCR using pAV2
[C. A. Laughlin et al, Gene, 23: 65-73 (1983)] as
template [nucleotide numbers 365-538 of SEQ ID NO: 1];

a CMV immediate early enhancer/promoter [Boshart et
al, Cell, 41:521-530 (1985); nucleotide numbers 563-1157
of SEQ ID NO: 1],

an SV40 intron (nucleotide numbers 1178-1179 of SEQ
ID NO: 1),

an *E. coli* lacZ cDNA (nucleotide numbers 1356 - 4827
of SEQ ID NO: 1),

an SV40 polyadenylation signal (a 237 Bam HI-BclI
restriction fragment containing the cleavage/poly-A
signals from both the early and late transcription units;
nucleotide numbers 4839 - 5037 of SEQ ID NO: 1) and

the 3' AAV ITR, obtained from pAV2 as a SnaBI-BglII
fragment (nucleotide numbers 5053 - 5221 of SEQ ID NO:
1).

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With reference to Fig. 1, two Bam HI sites are
present in the double-stranded vector sequence. The
first is located in the SV40 intron at bp position 875
and the second lies between the lacZ DNA and the SV40
5   polyadenylation signal at bp position 4469. Therefore,
digestion of the double-stranded sequence with BamHI
releases a fragment of 3595 bp in length. The location
of a cDNA probe that can be used to detect the internal
BamHI fragment, as well as full-length vector, is also
10 shown.

The rAAV.CMVlacZ virus was purified using standard
techniques [see, e.g., K. F. Kozarsky et al, J. Biol.
Chem., 269:13695-13702 (1994)]. Stocks of rAAV used in
the following examples were tested to ensure the absence
15 of replication competent wild-type AAV and helper
E1-deleted adenovirus as follows.

293 cells were seeded in chamber slides and
co-infected with wild-type adenovirus and an aliquot of
the rAAV vector stock. Twenty hours post-infection,
20 cells were fixed and incubated with a mouse monoclonal
antibody against AAV capsid proteins (American Research
Products). Antigen-antibody complex was detected with a
FITC-conjugated secondary antibody. A positive signal
was scored as one infectious AAV unit. Contaminating
25 helper adenovirus was measured by infecting 293 cells
with an aliquot of the rAAV vector stock and staining for
alkaline phosphatase reporter expression. The helper
adenovirus is E1-deleted and contains a human placenta
alkaline phosphatase cDNA under the transcriptional
30 control of the CMV promoter. Replication-competent wild-
type AAV or adenovirus helper was not detected in the
highly purified preparations of rAAV.

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Example 2 - rAAV Stably Transduces Skeletal Muscle in Vivo

rAAV was administered with and without an E2a-
deleted adenovirus, which was intended to enhance
5   transduction. Animal procedures were approved by the
Institutional Animal Care and Use Committee (IACUC) of
the Wistar Institute.

Briefly, five week old female C57BL/6 mice (Jackson
Laboratories, Bar Harbor, Maine) were anesthetized with
10   an intraperitoneal injection of ketamine (70 mg/kg) and
xylazine (10 mg/kg) and a subsequent 1 cm lower extremity
incision was made. Samples of rAAV.CMVLacZ ($1x10^9$ vector
genomes) in 25 μl of HEPES-Buffered-Saline (HBS, pH 7.8)
or rAAV.CMVlacZ supplemented with an adenovirus E2a
15   mutant d1802 [S. A. Rice and D. L. Klessig, J. Virol.,
56:767-778 (1985)] ($5x10^{10}$ A_{260} particles, $1x10^8$ pfu) just
prior to injection, were injected into the tibialis
anterior muscle of each leg using a Hamilton syringe.
Incisions were closed with 4-0 Vicryl suture.   To
20   analyze transgene expression, animals were necropsied at
various time points post-injection and injected muscle
was excised with a scalpel. Tissue was placed on a drop
of OTC embedding compound, snap-frozen in liquid
nitrogen-cooled isopentane for seven seconds, and
25   immediately transferred to liquid nitrogen. Analysis of
tissue at each time point represented a minimum of 6
injection sites (i.e., bilateral sampling from at least 3
animals).

For histochemical analysis, frozen muscle was
30   segmented laterally into two equal halves, generating a
cross-section face of the tissue. Both halves of the
tissue were serially sectioned (6 μm). For X-gal
histochemistry, sections were fixed in a freshly prepared
0.5% glutaraldehyde solution in PBS and stained for
35   β-galactosidase activity as described [K.J. Fisher et al,

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J. Virol., _70_:520-532 (1996)]. Sections were
counterstained in neutral red solution and mounted.

When adenovirus was used as a helper for the rAAV,
X-gal histochemistry analysis revealed high level
5   transduction of muscle fibers by day 17 associated with
substantial inflammation. Surprisingly, however, animals
that received rAAV in the absence of adenovirus helper
demonstrated levels of transduction that exceeded those
found in the presence of adenovirus. These high levels
10   have persisted without apparent diminution for 180 days.

Example 3 - rAAV Genome Integrates with High Efficiency
as Truncated Head-to-Tail Concatamers

In order to characterize the molecular state of the
stabilized rAAV genome, Southern blot analysis of DNA
15   from skeletal muscle harvested from mice injected as
above was performed. Models in which the rAAV genome
persists either as an episomal double-stranded genome
such as those formed during lytic infection, or as an
integrated provirus resembling latent infection were
20   considered.

Briefly, low molecular weight DNA (Hirt) (see Part A
below) and high molecular weight genomic DNA (see Part B
below) was isolated from mouse muscle at selected time
points. DNA samples were resolved on a 1% agarose gel and
25   electrophoretically transferred to a nylon membrane
(Hybond-N, Amersham). The blot was hybridized with a
^{32}P-dCTP random-primer-labeled restriction fragment
isolated from the lacZ cDNA.

A.   Detection of Episomal Double-Stranded Genome
30   To detect nonintegrated forms of the rAAV
genome, Hirt extracts of transduced muscle DNA were
analyzed by hybridization with a ^{32}P-labeled cDNA that
maps to the probe sequence shown in Fig. 1. The Hirt DNA
samples (15 μl, equivalent to 15 mg tissue) were

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extracted from muscle harvested on day 8, 17, 30, and 64
post-injection.

DNA from a cultured cell line infected with
rAAV in the presence of adenovirus was analyzed. The
5   analysis of Hirt extracts from that cell line
demonstrated the presence of both single-stranded and
monomeric double-stranded forms of the virus. However,
Hirt extracts of muscle transduced with rAAV alone
demonstrated the single-stranded genome by day 8 that
10   diminished to undetectable levels by day 64. Double-
stranded forms of rAAV were never detected in the Hirt
extracts even when the filters were over-exposed. This
indicates that the single-stranded rAAV genome is
efficiently transferred into cells of skeletal muscle;
15   however, it is not converted to transcriptionally active
episomal forms.

    B.   Detection and Characterization of Integrated
Proviral DNA

        To detect integrated proviral DNA, additional
20   hybridization studies were performed with total cellular
DNA harvested from transduced skeletal muscle 64 days
post-infection. Genomic DNA (10 μg, equivalent to 18 μg
tissue) was digested with BamHI or HindIII, a restriction
enzyme that does not cut proviral DNA. As expected,
25   HindIII digestion resulted in a smear after gel
fractionation and hybridization to a virus specific
probe. However, when genomic DNA was digested with
BamHI, which cuts twice within the provirus, a discrete
band of the predicted size of 3.6 kb was detected at an
30   abundance of approximately 1 proviral genome/diploid host
cell genome.

        The structure of the integrated provirus was
characterized using PCR analysis to delineate the
potential mechanisms of persistence. Previous studies of
35   wild type and rAAV have suggested different pathways of

DNA replication in the lytic and latent phases of the
viral life cycle. Specifically, in the presence of
helper virus, AAV replicates to form dimeric replicative
intermediates by a mechanism that results in the
synthesis of head-to-head or tail-to-tail concatamers.
This contrasts with latent infections where the
integrated proviral genome is characterized by head-to-
tail genomic arrays.

Genomic DNA from skeletal muscle was subjected
to PCR analysis to amplify junctions between AAV genomic
concatamers. A PCR method to detect integrated rAAV was
developed, based on data indicating that integrated forms
of rAAV are typically found as head-to-tail concatomers.
Specifically, oligonucleotide primers were synthesized to
allow selective PCR amplification across head-to-tail
junctions of two monomers of the AAV.CMVLacZ genome. The
sense-strand primer 005 (5'-ATAAGCTGCAATAAACAAGT-3'; SEQ
ID NO: 4) mapped to bp position 4584-4603 of the SV40
polyadenylation signal domain. The antisense-strand
primer 013 (5'-CATGGTAATAGCGATGACTA-3'; SEQ ID NO:2)
mapped to bp position 497-478 of the CMV promoter, while
the antisense-strand primer 017
(5'-GCTCTGCTTATATAGACCTC-3'; SEQ ID NO:3) mapped to bp
position 700-680 of the CMV promoter. If the ITRs are
retained intact, oligos 005 + 013 [SEQ ID NO:2] should
amplify a 797 bp fragment while oligos 005 and 017 [SEQ
ID NO: 3] should amplify a 1000 bp fragment. It is
important to emphasize that the predicted PCR product
sizes are based on the assumption that provirus junctions
contain two ITR copies. Amplification across a junction
that has fewer than two copies will therefore generate a
PCR product that is proportionally smaller in size.

PCR reactions were performed using 100 ng
genomic DNA template and primer concentrations of 0.5 μM.
The thermocycle profile was 94° C 1 min, 52° C 1 min, and

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72° C 1 min 30 sec for 35 cycles; the 94° C denaturation
step of the first cycle was 2 min, while the 72° C
extension step of the last cycle was 10 min. PCR products
were analyzed by agarose gel electrophoresis.

5   PCR reactions were conducted on genomic DNA
isolated from AAV.CMVLacZ transduced muscle harvested at
day 64 post-infection, as described above. Genomic DNA
from muscle injected with Hepes buffered saline (HBS) was
used as a negative PCR control. No amplification

10   products were detected when primers were used that should
span a head-to-head or tail-to-tail junction (data not
shown). However, when DNA from AAV.CMVlacZ transduced
muscle was analyzed with oligonucleotides 005 and 013 and
005 and 017, a smear was detected consistent with a

15   heterogenous population of head-to-tail concatamers
(Figs. 2A and 2B).

          PCR reactions were also conducted with genomic
DNA from cell lines that contain integrated AAV.CMVLacZ.
The provirus structure of these clones has been

20   determined by Southern blot analysis. Three cell lines
(10-3.AV5, 10-3.AV6, and 10-3.AV18) each of which contain
at least two monomer copies of integrated AAV.CMVLacZ
arranged head-to-tail were identified. Based on the size
of the PCR products (a 720 bp product using primer set

25   005-013, and a 930 bp product using primer set 005-017),
two clones 10-3.AV5 and 10-3.AV6 likely contain 1.5
copies of AAV ITR at the junction. Another clone 10-
3.AV18 contains a large deletion that encompasses the AAV
ITRs producing a 320 bp product using primer set 005-013

30   and a 500 bp product using primer set 005-017. Another
cell line 10-3.AV9 contains a single monomer copy of
integrated AAV.CMVLacZ according to Southern blotting,
and appears to be confirmed by the absence of a PCR
product.

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Thus, analysis of DNA from rAAV infected cell
lines selected for stable transduction revealed distinct
bands smaller than that predicted for an intact head-to-
tail concatamer.

5   D.   Structural Analysis

Detailed structural analyses of the proviral
junctions recovered from skeletal muscle DNA was
performed by subcloning from the PCR reaction (Fig. 3)
followed by restriction analysis (Figs. 4A-4G).

10   Particularly, PCR products from one of the muscle samples
BL.11 obtained as described above were directly ligated
into commercially available plasmid pCRII in which the
insert was flanked by EcoRI sites. Commercially
available competent bacterial strain TOP10 F' were

15   transformed with the ligation reactions. In effect, this
procedure results in a plasmid library of PCR products.
The library was plated at a density to give well isolated
colonies and screened by overlaying with a nylon membrane
and hybridizing with a ^{32}P-labeled fragment corresponding

20   to the CMV promoter/enhancer. Putative positive clones
were grown overnight in small-scale cultures (2 ml).

Plasmid DNA from six representative clones was
extracted from the small-scale cultures and digested with
either EcoRI to release the entire PCR product or with

25   SnaBI as a diagnostic indicator. Digestion with SnaBI
should release a 306 bp fragment (SnaBI 476 to SnaBI 782)
spanning the CMV promoter. The release of a second
fragment mapping to the ITR junction (SnaBI 142 to SnaBI
476) is contingent on rearrangements that occur during

30   formation of the concatomer, and could therefore range in
size from 334 bp (2 complete ITR copies) to 0 bp if the
ITRs have been deleted.

The PCR product from cell line 10-3.AV5
believed to contain 1.5 copies of AAV ITR (10-3.AV5) was

35   also cloned into pCRII and digested with the indicated

enzyme. This sample serves as a positive control for the diagnostic SnaBI digestion. Digestion of this sample with EcoRI correctly releases the 730 bp PCR fragment, as well as a secondary doublet band approximately 500 bp in

5 size. This secondary band is believed to be an artifact due to secondary structure that develops in the 1.5 copies of AAV ITR during replication in bacteria. Digestion of the positive control with SnaBI releases the diagnostic 306 bp fragment from the CMV promoter and a

10 250 bp fragment that maps to the ITR junction.

Digestion of the six individual clones with EcoRI and SnaBI indicated deletions of variable lengths were present in all recovered junctions and largely confined to ITRs at the junctions. Sequence analyses

15 further indicated that most deletions spanned portions of both ITRs at the junctions without involving contiguous viral DNA.

E.   <u>Fluorescence in situ hybridization (FISH)</u>
<u>Analysis</u>

20   FISH was performed on cryosections of skeletal muscle to characterize the distribution of proviral DNA within the injected tissue. Small 4-5 mm pieces of muscle from treated or control mice were embedded in OTC and quickly frozen in liquid isopentane cooled with

25 liquid nitrogen. Frozen sections, 10μ thick were cut on a cryomicrotome. Sections were mounted, fixed (Histochoice) and processed for fluorescence by *in situ* hybridization using a previously described protocol [E. Gussoni et al, <u>Nat. Biotech.</u>, <u>14</u>:1012-1016 (1996)].

30 Adjacent sections were stained for β-galactosidase activity to identify *lacZ* positive areas in muscle bundles.

For quantifying FISH signal, *lacZ* positive areas (as determined by staining adjacent sections for

35 β-galactosidase activity) were examined under a Nikon

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microphot FxA microscope equipped with epifluorescence.
The total number of individual muscle fibers
corresponding to *lacZ* positive area in the section were
counted under a standard phase contrast mode. The same
5   area was then examined under fluorescence microscopy
using the appropriate filter package for rhodamine
isothiocynate. The number of muscle cell nuclei showing
punctate staining was recorded. Each positive nucleus
was examined under phase contrast to ascertain that it
10 came from a muscle fiber. For controls, *lacZ* negative
areas (areas in the same sections that lacked
β-galactosidase activity, or mock transfected muscle
sections) were examined and quantified in a similar
manner.

15   For confocal microscopy, sections were observed
under an oil immersion objective lens (100X) on a Leica
confocal laser microscope equipped with Krypton-Argon
laser (Leica lasertechnik, GmbH), TSC and Voxel View
Silicon graphics central work stations. Images observed
20 under rhodamine channel were also sequentially observed
under differential interference contrast to confirm the
location of fluorescence signal with muscle cell nuclei.
The differential contrast and fluorescence images were
then sequentially superimposed on a TCS central work
25 station and were transferred to a Silicon graphics work
station for image processing. Processed images were
stored and printed using Photoshop software.

         Serial sections were alternatively stained for
β-galactosidase activity to identify transgene expressing
30 muscle fibers and hybridized with a biotinylated proviral
probe to localize the distribution of the proviral
genome. A discrete fluorescent signal was detected in
some nuclei of β-galactosidase expressing muscle fibers.
A survey of three serial sections revealed hybridization
35 in 53/1006 (5.3%) nuclei of β-galactosidase expressing

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fibers and 0/377 nuclei of fibers not expressing β-
galactosidase. Hybridization was not detected in tissues
from uninfected animals (data not shown).

The ability to detect viral genomes by FISH added
5   another dimension to the analysis. Single foci of
hybridization was detected in 5% of all nuclei contained
within muscle fibers expression β-galactosidase. It is
possible that this is an underestimate of transduced
nuclei, because of limitations in sensitivity of this
10   technique especially for target sequences less than 12 kb
in size [B.J. Trask, Trends Genet., 7:149-154 (1991)].
The implications of the FISH analysis are interesting.
The presence of 1 proviral genome/diploid myoblast
genome, as measured by Southern, together with the FISH
15   result that demonstrated 5% of nucleic acids harboring an
AAV genome would predict that the average concatamer
minimally comprises ten proviral genomes. These studies
show β-galactosidase enzyme activity that extends far
beyond the site of a vector transduced nucleus,
20   suggesting an extended nuclear domain of at least 10 μm.
This is consistent with previous work which documented
extended nuclear domains for cytostolic proteins [H.M.
Blau et al, Adv. Exp. Med. & Biol., 280:167-172 (1990].
An extended nuclear domain of transgene expression within
25   the syncytial structure of the muscle fibers is important
to applications of gene therapy for several reasons. The
net yield of recombinant protein from a transduction
event may be higher in a syncytium where the distribution
of protein is less constrained by membrane barriers.
30   Furthermore, this system has advantages in vector systems
that require expression of multiple recombinant proteins
such as those with inducible promoters [J.R. Howe et al,
J. Biol. Chem., 270:14168-14174 (1995); V. M. Rivera et
al, Nat. Med., 2:1028-1032 (1996)]. In muscle,

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coexpression of recombinant proteins does not require
cotransduction with a single nucleus because of the
extensive network of overlapping domains.

Example 4 - Transgene Directed Immune Responses are
Minimized when Helper-Free rAAV is Used for Muscle-
Directed Gene Delivery

The stability of lacZ expression in muscle cells
achieved from a lacZ-containing rAAV vector administered
in the absence of helper virus was surprising in light of
previous work which demonstrated destructive immune
responses mounted against ß-galactosidase expressed ·from
adenoviral vectors in muscle fibers. Transgene-specific
immune responses were studied further by measuring the
serum levels of anti-ß-galactosidase antibodies using
Western analysis.

Blood was drawn from C57BL/6 and ROSA-26 mice
(Jackson Laboratories, Bar Harbor, ME) necropsied on day
30 after virus injection and serum was collected.
ROSA-26 is a transgenic line that carries the *E. coli*
ß-galactosidase cDNA and was developed on a 129
background. Serum was also harvested from C57BL/6 mice
that received an intramuscular injection of either a
recombinant LacZ adenovirus (H5.010CMVLacZ, 5 x 10^8 pfu
in 25 μl of HBS) or the recombinant AAV.CMVLacZ (as
described above). Both vectors express *E.coli* ß-
galactosidase from a CMV-driven minigene. Aliquots (5
μg) of purified ß-galactosidase from E. coli (Sigma) were
resolved on a 10% SDS polyacrylamide gel (5 mg/lane) and
electrophoretically transferred to a nitrocellulose
membrane (Hybond-ECL, Amersham). The blot was incubated
with blotto [5% nonfat milk, 50 mM Tris-HCl (pH 8.0), 2
mM CaCl, and 0.05% Tween-20] at room temperature for 2
hours to block available sites. Individual lanes were
cut and incubated with serum (diluted 1:200 in blotto)

for 1 hour at room temperature. Localization of
antigen-antibody complex was accomplished by adding goat
anti-mouse horseradish peroxidase conjugate, followed by
ECL detection (Amersham). The cut lanes were reassembled

5   on a mylar sheet prior to addition of ECL reagent and
film documentation.

Intramuscular injection of the H5.010CMVlacZ E1
deleted adenovirus into skeletal muscle of C57BL/6 mice
resulted in substantial ß-galactosidase antibody

10   accumulation in serum that did not occur in MHC-identical
transgenic animals carrying an inserted lacZ gene which
are immune tolerant to β-galactosidase. Significantly,
neither C57BL/6 nor *lacZ* transgenic animals developed
antibodies to ß-galactosidase after intramuscular

15   injection with AAV.CMVlacZ.

Example 5 - Comparative Studies of Adenoviral and AAV
Vectors in Muscle Cells

Studies of the biology of muscle-directed gene
transfer mediated by recombinant AAV and adenovirus

20   demonstrate that adenoviruses, but not AAV, infect
antigen presenting cells (APCs), which elicit a cascade
of immunological responses leading to destructive
cellular and humoral immunity.

An experimental paradigm was constructed to define

25   the specific differences in host responses to skeletal
muscle-directed gene transfer with recombinant AAV and
adenovirus. The goal was to delineate differences in the
biology of these vector systems that lead to preferential
immunologic activation directed against a transgene

30   product (i.e., β-galactosidase) when expressed from a
recombinant adenoviral vector, but not an AAV vector.

The general approach was to inject a *lacZ* expressing
AAV into the right leg of a mouse. This has been shown

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in the examples above to confer efficient and stable gene
expression. In other experimental groups, the animals
receive rAAV in addition to various combinations of
vectors and cells in order to define components of the
5   immune response directed against Ad that lead to
destructive cellular and humoral immunity. The effects of
these experimental manipulations were followed by
assessing their impact on the stability of the rAAV
engrafted muscle fibers, as well as measuring other
10   immunologic parameters. Any intervention that elicits
immunity to β-gal in muscle fibers can be detected by
assessing the stability of transgene expression in the
AAV-transduced muscle, and the development of
inflammation.
15   Four experimental groups were developed for this
study as summarized below. Virus was injected into mice
using techniques substantially similar to those described
in Example 2 above, i.e., virus was suspended in
phosphate-buffered saline and injected directly into the
20   tibialis anterior muscles. When the animals were
necropsied, muscle tissues were snap-frozen in liquid
nitrogen-cooled isopentane and sectioned at 6 μm
thickness, while serum samples and the draining inguinal
lymph nodes were harvested for immunological assays.
25   Lymphocytes were harvested from inguinal lymph nodes
and a standard 6 hr [51]chromium (Cr)-release assay was
performed essentially as described below, using different
ratios of effector to target cells (C57SV, H-2[b]) in 200 μl
DMEM in V-bottom 96-well plates. Prior to mixing with
30   the effector cells, target cells were either infected
with an adenovirus expressing alkaline phosphatase
(Ad*ALP*) or stably transduced with a lacZ-expressing
retrovirus, pLJ-lacZ, labeled with 100 μCi of ^{51}Cr and
used at 5 x 10^{3} cells/well. After incubation for 6 hr,

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aliquots of 100 μl supernatant were counted in a gamma
counter. The results for groups 1-3 are provided in
Figs. 5A-5C.

Frozen sections (6μm) were fixed in methanol and
5   stained with anti-CD4 and anti-CD8 antibodies.
Morphometric analysis was performed to quantify the
number of CD8+ cell and CD4+ cells per section.

The cytokine release assay was performed essentially
as follows. Lymphocytes were restimulated for 40 hr with
10   β-galactosidase, purified AAV, or adenovirus type 5.
Cell-free supernatants (100 μl) were assayed for the
secretion of IL-10 and IFN-γ. Proliferation was measured
72 hr later by a 8 hr ^{3}H-thymidine (0.50 μCi/well) pulse.
The results for the four groups are provided in Figs. 6A
15   and 6B.

The neutralizing antibody assay was performed
essentially as follows. Mouse serum samples were
incubated at 56°C for 30 min to inactivate complement and
then diluted in DMEM in two-fold steps starting from
20   1:20. Each serum dilution (100 μl) was mixed with β-
galactosidase or adenovirus type 5. After 60
minincubation at 37°C, 100 μl of DMEM containing 20% FBS
was added to each well. Cells were fixed and stained for
β-galactosidase expression in the following day. All of
25   the cells stained blue in the absence of serum samples.
The results for the four groups are provided in Figs. 6A
and 6B.

Group 1 mice received AAV.CMV$lacZ$, produced as
described in Example 1, in the right leg with no other
30   intervention. Transduction with AAV.CMV$lacZ$ alone led to
high levels of stable gene transfer (evident even at 28
days) without infiltration of lymphocytes. No activation
of CD8 T cells was detected (Fig. 5). Nor were antigen-
specific, CD4$^+$ T cells [i.e., viral or β-galactosidase

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(Figs. 6A and 6B)] detected. Antibodies were not
generated to β-galactosidase or adenovirus (Figs. 7A and
7B).

Group 2 mice received AAV.CMV*lacZ* in the right leg

5   and adenovirus expressing *lacZ* (H5.010CMVlacZ) in the
left leg. The goal of this group was to determine if the
immunologic response to the Ad-infected muscle fibers was
systemic, as demonstrated by its impact on the biology of
the contralateral AAV.CMV*lacZ* transduced leg.

10   Apparently, the adenoviral lacZ treatment induced an
immune response to β-galactosidase that led to the
destruction of the AAV *lacZ* transduced fibers. Not
surprisingly, this was associated with infiltration of
both CD4 and CD8 T cells into the AAV transduced leg, and

15   the activation of cytotoxic T lymphocytes to both
adenoviral and β-galactosidase antigens (Fig. 8).
Activated CD4 T cells specific for AAV, Ad, and β-gal
antigens and antibodies specific for adenovirus and β-
galactosidase were also observed.

20   Group 3 animals received a mixture of AAV.CMV*lacZ*
and Ad BglII in the right leg. AdBglII is an E1-deleted
adenovirus that expresses no recombinant gene. The goal
of this group was to determine if the adenovirus provides
an adjuvant effect which would elicit immunity to AAV to

25   *lacZ* in this setting. This did not lead to loss of
transgene expression, although there was substantial
infiltration of CD8 T cells and some activation of CD4 T
cells to viral antigens, but not to β-galactosidase
(Figs. 6A-6B). As expected, antibodies were generated

30   towards adenovirus but not towards β-galactosidase (Figs.
7A-7B).

Group 4 animals received AAV.CMV*lacZ* in the right
leg and were adoptively transferred with antigen
presenting cells harvested from naive animals and

35   infected *ex vivo* with adenovirus.

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These animals mounted a vigorous and effective
immunologic response to β-gal, as demonstrated by the
loss of transgene expression, and the massive
infiltration of CD8 and CD4 T cells. CD4 T cells were
5   activated to β-gal in this experiment as shown in Figs.
6A-6B, and anti-β-galactosidase antibodies generated, as
shown in Figs. 7A-7B.

Example 6 - Transduction of a purified rAAV vector
containing Factor IX into skeletal muscle cells and
10   expression of F.IX at therapeutically useful levels and
without eliciting a cytotoxic immune response.
The data provided in this example demonstrates that
the method of this invention provides prolonged
expression of a therapeutic transgene, F.IX, in both
15   immunocompetent and immunoincompetent subjects in the
absence of an immune response cytotoxic to the transduced
cells. Further, the protein levels achieved in the serum
of immunoincompetent animals are adequate to achieve a
therapeutic effect. Thus, prolonged expression of human
20   F.IX in muscle cells via rAAV vectors in
immunoincompetent patients, such as those with hemophilia
B, is useful to deliver F.IX to patients with that
disease.
A.   Preparation of purified rAAV
25   The rAAV vector used in the following in vivo
experiments carries an expression cassette containing the
human F.IX cDNA including a portion of Intron I under
transcriptional control of the cytomegalovirus (CMV)
immediate early gene promoter/enhancer and SV40
30   transcription termination signal. The vector, which
contains this expression cassette flanked by AAV ITR
sequences, and which completely lacks AAV protein coding
sequences, was constructed as follows.

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        Recombinant AAV was generated by co-transfection of
a F.IX *cis* plasmid (pAAV-FIX) and the trans-acting
plasmid pAAV/Ad [A.W. Skulimowski and R.J. Samulski,
Method. Mol. Genet., 7:7-12 (1995)] into human embryonic
5   kidney (293) cells infected with an E1-deleted adenovirus
as described by Fisher et al., J. Virol., 70:520-532
(1996). pAAV-FIX was derived from psub201 [Skulimowski
and Samulski cited above] and contains the CMV
promoter/enhancer, the human F.IX coding sequence
10 including 1.4-kb fragment of Intron I [S. Kurachi et al,
J. Biol. Chem., 270:5276-5281 (1995)], and the SV40
polyadenylation signal, flanked by AAV ITR sequences.
The AAV *rep* and *cap* gene functions were supplied in *trans*
by pAAV/Ad. The E1-deleted adenovirus contained a ß-
15 galactosidase (LacZ) or alkaline phosphatase (ALP)
reporter gene to trace potential contamination of rAAV
stocks with this helper virus. Cells were lysed 48 hours
after transfection by sonication, and the released rAAV
particles were purified by four rounds of CsCl density
20 gradient centrifugation as described by Fisher et al.,
cited above.

        The resulting rAAV-F.IX particles had a density
of 1.37-1.40 g/ml. The titer of the purified rAAV-F.IX
was determined by slot blot hybridization using a probe
25 specific to either the CMV promoter or Intron I sequences
and standards of pAAV-F.IX plasmid DNA of known
concentration. The ability of rAAV-F.IX to transduce
cells *in vitro* was confirmed by transducing growing HeLa
cells and measuring the concentration of hF.IX in the
30 culture supernatant 36 hours post-infection with an ELISA
specific to hF.IX [J. Walter et al, Proc. Natl. Acad.
Sci. USA, 93:3056-3061 (1996)]. rAAV-F.IX (10^{12}-10^{13}
genomes/ml) was stored at -79°C in HEPES-Buffered Saline,
pH 7.8, including 5% glycerol.

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Purified rAAV-F.IX routinely lacked detectable
amounts of contaminating adenovirus when analyzed by
transduction of 293 cells followed by staining for
alkaline phosphatase or β-galactosidase as described by
5   Fisher et al., cited above. Wild-type AAV was detected
at <1 infectious unit per 10^9 genomes of rAAV-F.IX. The
assay for wild-type AAV was as follows: 293 cells grown
on chamber slides were co-infected with adenovirus and
with aliquots of purified rAAV-F.IX and fixed for
10   immunofluorescence staining 24 hours post-infection. A
mouse monoclonal antibody against AAV capsid proteins
(American Research Products, Belmont, MA) served as a
primary antibody, and anti-mouse IgG (DAKO Corporation,
Carpinteria, CA) in a dilution of 1:40 as secondary
15   antibody.

B.   Introduction of rAAV into skeletal muscle
Mouse strains selected for intramuscular
injection with rAAV were C57BL/6 (Charles River
Laboratories, Wilmington, MA) and B6, 129, Rag 1 (Jackson
20   Laboratories, Bar Harbor, Maine). Female mice (4-6 week-
old) were anesthetized with an intraperitoneal injection
of ketamine (70 mg/kg) and xylazine (10 mg/kg), and a 1
cm longitudinal incision was made in the lower extremity.
AAV-F.IX ($2x10^{11}$ or $1x10^{10}$ vector genomes/animal in
25   HEPES-buffered saline, pH 7.8) was injected into the
tibialis anterior (25 μl) and the quadriceps muscle
(50μl) of each leg using a Hamilton syringe. Incisions
were closed with 4-0 Vicryl suture. Blood samples were
collected at seven-day intervals from the retro-orbital
30   plexus in microhematocrit capillary tubes and plasma
assayed for hF.IX by ELISA (part C below). For
immunofluorescence staining (part D below) and DNA
analysis (part F below), animals were sacrificed at
selected time points and injected and non-injected muscle
35   tissue was excised. Tissue was placed in OTC embedding

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compound, snap-frozen in liquid nitrogen-cooled
isopentane for seven seconds, and immediately transferred
to liquid nitrogen.

    C.   <u>Detection of human F.IX by ELISA</u>

5   Human F.IX antigen in mouse plasma was
determined by ELISA, as described by Walter et al., cited
above. This ELISA did not cross-react with mouse F.IX.
All samples were measured in duplicate. Protein extracts
from injected mouse muscle were prepared by maceration of
10   muscle in phosphate buffered saline (PBS) containing
leupeptin (0.5 mg/ml) followed by sonication. Cell
debris was removed by microcentrifugation, and 1:10
dilutions of the protein extracts were assayed for hF.IX
by ELISA. Extracts from rAV.CMVLacZ (see Example 1
15   above)-injected muscle were used as negative controls.
Protein concentrations were determined with the BIORAD
assay (Bio-Rad, Hercules, CA).

    D.   <u>Immunofluorescence staining</u>

        In order to perform immunofluorescence staining
20   of tissue sections, cryosections of muscle tissue (6μm)
were fixed for 15 minutes in 3% paraformaldehyde in PBS,
pH 7.4, rinsed in PBS for 5 minutes, incubated in
methanol for 10 minutes, washed three times in PBS, and
then blocked in PBS/3% bovine serum albumin (BSA) for 1
25   hour. Sections were subsequently incubated overnight
with an affinity purified goat anti-human F.IX antibody
(Affinity Biologicals) that was diluted 1:1000 in PBS/1%
BSA. After three washes (10 minutes each) in PBS/1% BSA,
the secondary antibody was applied for 90 minutes (FITC-
30   conjugated rabbit anti-goat IgG, DAKO Corporation,
diluted 1:200 in PBS/1% BSA). After three additional
washes in PBS/1% BSA, sections were rinsed in distilled
water, air-dried and mounted with Fluoromount G mounting
media (Fisher Scientific). All incubation steps were at
35   room temperature, except for incubation with the primary

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antibody (4°C). The same protocol was applied when
sections were stained with rabbit anti-human collagen IV
as primary antibody (Chemicon, Temecula, CA) in a 1:500
dilution and FITC conjugated anti-rabbit IgG (DAKO
5   Corporation) as secondary antibody. For co-localization
studies, a goat anti-hF.IX antibody conjugated to FITC
(Affinity Biologicals) was applied simultaneously with
the anti-collagen IV antibody, and rhodamine-conjugate
anti-rabbit IgG (Chemicon) was used to detect collagen
10   IV-antibody complexes. Fluorescence microscopy was
performed with a Nikon FXA microscope.

     E.   Tests for circulating antibody against hF.IX
          Plasma samples of C57BL/6 mice intramuscularly
injected with AAV-F.IX were tested for the presence of
15   antibodies against hF.IX using the ELISA. Microtiter
plates were coated with human F.IX (1 µg/ml in 0.1M
NaHCO$_3$, pH 9.2). Dilute plasma samples (1:16) were
applied in duplicate, and antibodies against hF.IX
detected with horseradish peroxidase conjugated anti-
20   mouse IgG (Zymed, San Francisco, CA) in a dilution of
1:2000. Buffer conditions were as described in Walter et
al, cited above. Anti-hF.IX levels were estimated by
comparison of absorbance values with monoclonal mouse
anti-hF.IX (Boehringer Mannheim) diluted to a final
25   concentration of 1 µg/ml. Western blots to demonstrate
the presence of anti-hF.IX were performed as outlined by
Dai et al., Proc. Natl. Acad. Sci. USA, 92:1401-1405
(1995), except that a horseradish peroxidase conjugated
goat anti-mouse IgG antibody (Boehringer Mannheim) was
30   used as secondary antibody, thereby allowing the
detection of hF.IX-antibody complexes with ECL reagent
(Amersham). Dilution of mouse plasma were 1:500.

     F.   DNA analyses
          Genomic DNA was isolated from injected muscle
35   tissue as described for mammalian tissue by Sambrook et

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al., <u>Molecular Cloning: A Laboratory Manual</u>, Cold Spring
Harbor Press, Cold Spring Harbor, NY (1989). As described
in Example 3 of the application, PCR reactions were
carried out in order to amplify head-to-tail junctions of
5 rAAV tandem repeats. The forward primer 005 [SEQ ID
NO:4] anneals the SV40 polyadenylation signal (bp
position 8014-8033), and reverse primers 013 [SEQ ID NO:
2] and 017 [SEQ ID NO: 3] bind to the CMV promoter (bp
position 4625-4606 and 4828-4809). PCR reactions were
10 performed using 100 ng genomic DNA in a total reaction
volume of 100 μl including 1.5 mM $MgCl_2$, and 0.5 μM of
primer pair 005/013 or 005/017. After an initial
denaturation step (94°C for four minutes), 35 cycles of
the following profile were carried out: denaturation at
15 94°C for four minutes), 35 cycles of the following
profile were carried out: denaturation at 94°C for 1
minutes, annealing at 52°C for 1 minute, extension at
72°C for 90 seconds (10 minutes during the final cycle).
PCR products were cloned (for DNA sequence analysis)
20 using the T/A cloning kit (Invitrogen, San Diego, CA).
Southern blot hybridizations were performed using ^{32}P-
dCTP random primed labelled probes specific for the CMV
promoter (for hybridization to PCR fragments) or for
intron I of hF.IX as present in rAAV-F.IX (for
25 hybridization to genomic mouse DNA).

         G.   <u>Results of expression of hF.IX in</u>
<u>immunocompetent mice</u>

         Immunocompetent C57BL/6 mice were injected
intramuscularly with rAAV-hF.IX and the animals
30 sacrificed one month post-injection. An ELISA on
protein extracts from injected muscles (tibialis anterior
and quadriceps) demonstrated the presence of 1.8-2.1 ng
hF.IX/mg tissue (40-50 ng hF.IX/mg protein). The
expression of hF.IX in muscle tissues was confirmed by
35 immunofluorescence studies on tissue sections.

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Immunocompetent C57BL/6 mice were injected
intramuscularly with rAAV-hF.IX and the animals
sacrificed three months post-injection. Factor IX was
not detected in uninjected muscle. Factor IX was not
5   detected in muscle injected with a control, rAAV-lacZ.
Expression of human F.IX was detected in muscle fibers of
C57BL/6 mice three months post-injection (3.3×10^{10} vector
genomes per injection site; magnification 200x). Note
that F.IX is present not only in the muscle fibers
10   themselves, but in the interstitial spaces between the
fibers as well where it appears to accumulate.

Interestingly, this staining pattern was
identical to that seen with a polyclonal antibody against
human collagen IV, which also stained the interstitial
15   space. A stain of muscle one month post infection with
rAAV-hF.IX (3.3×10^{10} genomes per injection site)
stained well with antibody to human collagen IV (data not
shown). Collagen IV has recently been identified as a
binding protein for human F.IX [W.-F. Cheung et al, Proc.
20   Natl. Acad. Sci. USA, 93:11068-11073 (1996)].

Initial experiments in immunocompetent mice
demonstrated that, despite high levels of gene transfer
and stable expression of hF.IX in injected muscle, it was
not possible to detect significant amounts of hF.IX in
25   the circulation by ELISA. See, Fig. 8. Further
experiments demonstrated that the animals had developed
high-titer antibodies against the circulating foreign
protein. For example, when the same plasma samples were
tested for antibodies against human F.IX, a strong
30   antibody response was seen in all injected animals
starting at day 11 post-injection. See Fig. 9. Using
Western blot analysis, high levels of circulating
antibody were found to have persisted for the duration of
the experiment.

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     This finding contrasted with our previously
reported experience, in which a different route of
administration, i.e., intravenous injection, of a
different vector, i.e., an adenoviral vector expressing

5   hF.IX, effected a different immune response, i.e., did
not trigger formation of neutralizing antibodies against
hF.IX (Walter et al, cited above)].

     Levels of protein expression required to induce
antibody formation however are quite low. The Western

10   blot assay is somewhat less sensitive than an ELISA but
documents what appears to be a rising antibody titer
beginning 18 days after injection. Thus in the
immunocompetent animals, the absence of detectable hF.IX
expression at initial time points is a result of the

15   biology of rAAV expression in muscle, whereas the
subsequent absence of detectable F.IX in the circulation
results from the production of antibodies to the foreign
protein. Nevertheless the serum antibody response was
not associated with cytotoxic immune response directed

20   against the transgene-expressing cells. In fact, neither
inflammation nor extensive tissue damage was observed in
any of the tissue sections discussed above nor in
sections analyzed by H&E staining (data not shown). This
contrasts with the immune response elicited by injection

25   of skeletal muscle with recombinant adenovirus carrying a
transgene [Dai et al, cited above; Y. Yang et al, Hum.
Molec. Genet., 5:1703-1712 (1996); X. Xiao et al, J.
Virol., 70:8098-8108 (1996)].

    H.   The expression of hF.IX in immunodeficient mice

30   AAV-F.IX was delivered to muscles of Rag 1
mice, which are homozygous for a mutation in the
recombinase activating gene 1. These animals are
therefore functionally equivalent to severe combined
immunodeficiency (SCID) mice and do not produce mature B

35   or T cells. A dose of 2×10^{11} rAAV-hF.IX vector genomes

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per animal resulted in stable expression of hF.IX in
mouse plasma. See Fig. 10. Human F.IX was first
detectable by ELISA in the second week after the
injection and rose gradually thereafter. Plasma levels
5   in all animals reached a plateau of therapeutic levels of
F.IX five to seven weeks post-injection at 200 to 350 ng
hF.IX/ml mouse plasma. This level was maintained for the
duration of the experiment (four months post-injection).
When a total of 1×10^{10} rAAV-hF.IX vector genomes was
10   injected, expression was three- to four-fold lower, but
still reached therapeutic levels (>100 ng/ml) for some
animals. See, Fig. 11. These levels, which represent 4-
7% of normal circulating levels in plasma, are well
within a therapeutic range, and demonstrate that the
15   method of this invention is a feasible for the treatment
of hemophilia, a disease associated with
immunodeficiency.
        I.   Results of DNA Analysis
            Genomic DNA from injected muscle tissue was
20   isolated six to eight weeks post-injection. The presence
of introduced vector DNA was demonstrated by digestion
with EcoRV, which releases a 1.8-kb fragment from the
vector construct including the entire 1.4-kb intron I
sequence. A probe specific to intron I hybridized to
25   this fragment and did not cross-hybridize to mouse DNA
from an uninjected animal. Undigested DNA showed as
hybridization signal in the high molecular weight DNA.
Furthermore, PCR primers designed to amplify junction
sequences of head-to-tail concatamers of recombinant AAV
30   present in transduced cells (Fig. 12) successfully
amplified such sequences from muscle DNA isolated from
AAV-F.IX transduced tissue (tibialis anterior and
quadriceps of immunodeficient and immunocompetent
animals). The PCR products were visualized by Southern
35   blot hybridization with a probe specific to the CMV

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promoter/enhancer. Primer pair 005-013 produced
fragments that were 1.0-kb and smaller; primer pair 005-
017 amplified fragments that were 1.2-kb and smaller. As
expected, these PCR reactions resulted not in distinct
5   bands of the sizes noted above, but rather in a series of
amplification products with a maximum size as predicted,
because of imprecise joining of AAV genomes present in
these tandem repeats [S. K. McLaughlin et al, _J. Virol._,
_62_:1963-1973 (1988)]. The imprecise joining results from
10   variable deletions of ITR sequences at the junction sites
as confirmed by DNA sequencing of cloned PCR products
(data not shown).

        J.   PCR studies
             While head-to-head and tail-to-tail
15   arrangements of AAV genomes can occur during viral
replication [K. I. Berns, _Microbiol. Rev._, _54_:316-329
(1990)], head-to-tail arrays are more typically
associated with AAV that has been integrated into the
chromosomal DNA of the transduced cell during latent
20   infection [S. K. McLaughlin et al, _J. Virol._, _62_:1963-
1973 (1988); J. D. Tratschin et al, _Mol. Cell Biol._,
_5_:3251-3260 (1985); N. Muzcyka, _Current Topics in
Microbiology and Immunology_, _158_:97-129 (1992)].
             Southern blot data on undigested rAAV-injected
25   muscle cell DNA demonstrated that the rAAV DNA derived
from host cell genomic DNA six weeks after injection is
present as a high molecular weight species. The higher
signal intensity seen with the restricted DNA likely
results from an unmasking effect when the fragments are
30   separated from the bulk of genomic DNA [X. Xiao et al, _J._
Virol., _70_:8089-8108 (1996)]. This finding, the presence
of a hybridization signal for high molecular weight DNA,
is, like the PCR data, consistent with integrative events
occurring during transduction. The integration state of

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the rAAV discussed in this paragraph and in paragraph I
is also likely to contribute to the stability and
prolonged expression of the transgene.

Example 7 - Expression of ApoE using rAAV Administered to
5   a Skeletal Muscle Cell
         The following example demonstrates the prolonged
expression of another therapeutic transgene product,
apolipoprotein E (ApoE), a protein useful in the
treatment of atherosclerosis, by introduction into
10   skeletal muscle of a rAAV vector according to this
invention. Again, the absence of a destructive CTL
response permits the prolonged expression of the
transgene product.
         A.   Construction of the rAAV
15   Recombinant AAV vectors encoding the secreted
protein human ApoE were constructed in a manner similar
to that described above for F.IX. ApoE cDNA was excised
from a plasmid pAlterApoE3 (contributed by Dr. Rader's
laboratory, University of Pennsylvania) with XbaI
20   digestion, blunted and cloned into NotI digested pCMVLacZ
backbone (see the vector construction diagram of Fig.
13). A 2062 bp SmaI/SacI fragment from the lacZ gene in
pCMVLacZ was isolated and inserted into the SalI site of
the new plasmid as a stuffer. The ApoE minigene
25   cassette, which now contained the CMV promoter, ApoE cDNA
SV40 polyadenylation sequences and a 2062 bp stuffer, was
isolated by EcoRI/HindIII digestion (total length: 4.3
kb) and then ligated to an XbaI digested pSub201
backbone. The final product, designated pSubCMVApoE-
30   2062RO, was used as the cis plasmid in the production of
rAAV.ApoE production following the procedures described
above for rAAV.F.IX.

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B.   *In vitro analysis of ApoE expression by*
rAAV.ApoE

84-31 cells seeded in 6 well plates were
infected with 2 microliters of CsCl purified rAAV.ApoE in
5   2 ml Dulbeccos Modified Eagles Medium with 2% fetal
bovine serum.   The cells were kept at 37°C for 48 hours.
An aliquot of the supernatant was then removed from the
well for a Western blot analysis of ApoE.   The results
showed ApoE protein was clearly detectable in the
10   supernatant of 84-31 cells.infected with AAV.ApoE.

C.   *In vivo expression of rAAV.ApoE in ApoE*
knockout mice

2.5 to 5 X 10^{10} particles of rAAV.ApoE were
injected into anterior tibialis and quadriceps muscles on
15   each side of ApoE knockout mice (total particles per
mouse: 5 X 10^{10} to 5 x 10^{11}).   Prolonged local ApoE
expression was detectable by immunofluorescence at day 28
and day 120 post-injection even in the presence of plasma
anti-ApoE antibodies.

20   D.   Conclusion

This experiment demonstrates that intramuscular
injection of the vector, rAAV-ApoE, into Apo-E knockout
mice leads to muscle fiber transduction and secretion of
substantial quantities of the recombinant protein in the
25   circulation.   Prolonged expression of the transgene in
the muscle fiber in the absence of a destructive CTL
immune response was achieved, even though humoral
immunity was elicited to the secreted protein.

Example 8 - Transgene expression in a primate

30   A rhesus monkey was anesthetized, the forearm was
clipped and aseptically prepared and a 0.5 cm incision
was made in the skin over the tibialis anterior muscle.
The fascia was identified and the rAV.CMVLacZ (described
in Example 1) viral suspension (175 microliter of 10^2

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genomes/ml) was injected 5-7 mm deep into the fascia.
Fourteen days later, a muscle biopsy was removed, frozen
in OCT, sectioned and stained in X-gal. Tissue sections
were quantitatively analyzed using the Leica Z500MC Image
5   Processing and Analysis System interfaced with a Nikon
FXA Microscope. X-gal histochemistry revealed high level
ß-galactosidase expression in the majority of muscle
fibers in the area of the injection site. Twenty percent
of fibers expressed ß-galactosidase in a 224 mm^2 area in
10   the region of the injections. Based on the above-
described results with ApoE and F.IX, expression is
expected to be prolonged in the absence of a cytotoxic
immune response.   These data support that the
expression of a transgene according to this invention can
15   be duplicated in an animal other than a mouse, and
particularly in a primate animal.

Numerous modifications and variations of the present
invention are included in the above-identified
specification and are expected to be obvious to one of
20   skill in the art. Such modifications and alterations to
the processes of the present invention are believed to be
encompassed in the scope of the claims appended hereto.